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Ameba-Bacterium Relationship in Amebiasis

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INTRODUCTION

Human infection with the protozoan intestinal parasite *Entamoeba histolytica*, which causes amebiasis, usually begins with ingestion of the dormant form of the organism, the cyst, which is present in water or vegetables polluted with human fecal material (54). Studies of the life cycle of this parasite have shown that motile trophozoites emerge after digestion of the tough cyst wall (excystation) in the small intestine and migrate to the colon region, where they can proliferate in the microaerophilic conditions together with the resident bacterial flora (Fig. 1). In most cases, trophozoites in the intestine live as commensals, without causing any noticeable damage or discomfort to the host. In some instances, however, trophozoites display an aggressive behavior, attacking and invading the intestinal mucosa and causing dysentery. The parasites can also progress through blood vessels to the liver, brain, or lungs, where they may form life-threatening abscesses (extraintestinal lesions). Trophozoites which remain in the intestine exit the body together with the feces. Before leaving, however, many of them encyst and produce a quadrinucleated cell which is surrounded by a protective wall that can withstand the hostile environmental conditions encountered outside the host and enable propagation.

Epidemiological studies on the prevalence of *E. histolytica* infection indicate that over 480 million people in the world harbor the parasite, and every year about 10% of those infected show some sort of clinical symptoms (dysentery, intestinal invasion) (114). One of the theories for the disparity between the number of asymptomatic carriers and diseased individuals is that carriers are infected with nonpathogenic strains which are incapable of causing disease. On the other hand, in the absence of clear genetic evidence for strain differences, we should also examine other factors which may determine the virulence of *E. histolytica* in the human host and its occasional conversion from a harmless intestinal commensal to an aggressive invader.

The study of microbial interrelationships as a factor in microbial pathogenicity is an obscure area of microbiology which has lent itself to only limited scientific investigation. The possible role of symbiosis of associated microorganisms in the production of disease is largely unknown. The possibility that the indigenous or autochthonous flora of a host protects, and may in some instances help combat invasions by various microbes, is generally accepted (23), but the possibility that it may, in other instances, actually render the host susceptible to certain pathogens has received less consideration.

It is generally recognized that pathogenicity of *E. histolytica* is related in part to the association of amebae with suitable bacterial species. The definitive influence exerted by bacteria in the production of amebic dysentery was suspected already in 1891 (4), and since that time it has been frequently observed (14, 24, 83–85, 117, 119). The aim of this article is to try to critically review the available information on this subject and to discuss this poorly understood, yet intriguing, phenomenon.

CULTIVATION OF *E. HISTOLYTICA*: DEPENDENCE ON BACTERIA

In their natural environment, trophozoites of *E. histolytica* live in the colon region of the human intestine together with resident microbial flora, which under normal conditions is usually composed of a complex mixture of mostly anaerobic or microaerophilic bacteria (22, 23, 40, 105).

During the early work on in vitro cultivation of trophozoites of *E. histolytica*, Cleveland and Sanders (21) found that continuous propagation of amebae was impossible in the absence of living bacteria and trophozoites isolated from bacteriologically sterile liver abscesses of inoculated kittens required certain bacterial species for successful growth.

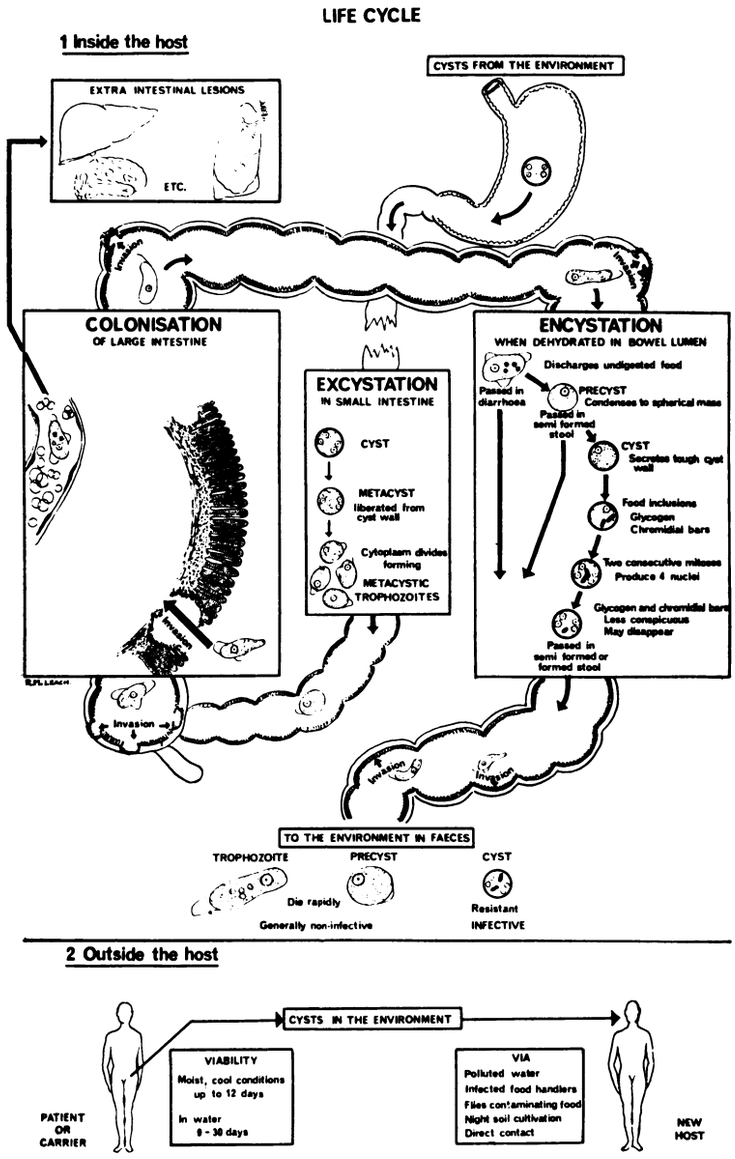

FIG. 1. Life cycle of *E. histolytica*.

Leon Jacobs' investigations reported in 1947 (44) opened a new era in the cultivation of *E. histolytica* by introducing penicillin to inhibit the growth of associated bacteria. These experiments demonstrated for the first time that metabolizing bacteria were not necessary for growth of *E. histolytica* and that growth, albeit poor, could be maintained in a medium containing heat-inactivated bacterial cells. Shaffer and his associates (106–108) extended Jacobs' investigations and, through the use of a penicillin-sensitive bacterium, *Clostridium symbiosum*, developed a monoxenic culture; this was used in the search for the elusive factors essential for multiplication of the ameba. These studies led to the finding that certain other cells could substitute for bacteria in the cultures. Amebae would grow well with *Trypanosoma cruzi* (52, 80) or with living cells of the chicken embryo (108). Even lethally irradiated bacteria could be used as additives to growth media (94). On the other hand, cell-free filtrates of bacterial, trypanosomal, or animal cell cultures were not able to replace the cells from the growth medium. The apparent dependency on metabolically active cells for the growth of *E. histolytica* was also assumed from in vivo observations whereby amebae sometimes disappeared from the intestine following treatment with bactericidal, but not amebicidal, drugs (15, 32). The most intriguing question at that time was what the bacteria furnished the ameba in culture. One concept suggested that the bacterial flora provides anaerobic conditions or redox potentials that are suitable for amebic growth (20, 43, 71). However, if this were the sole role the bacteria fulfilled, a number of reducing agents, gas mixtures, or culture filtrates in which bacteria had grown could have been used in their place, and none were shown to be successful (71). Another theory suggested that the products of bacterial metabolism are essential for the amebae (92). Since *E. histolytica* was known to develop in the liver in the absence of bacteria (95), it appeared that metabolic products of the bacteria could be replaced by

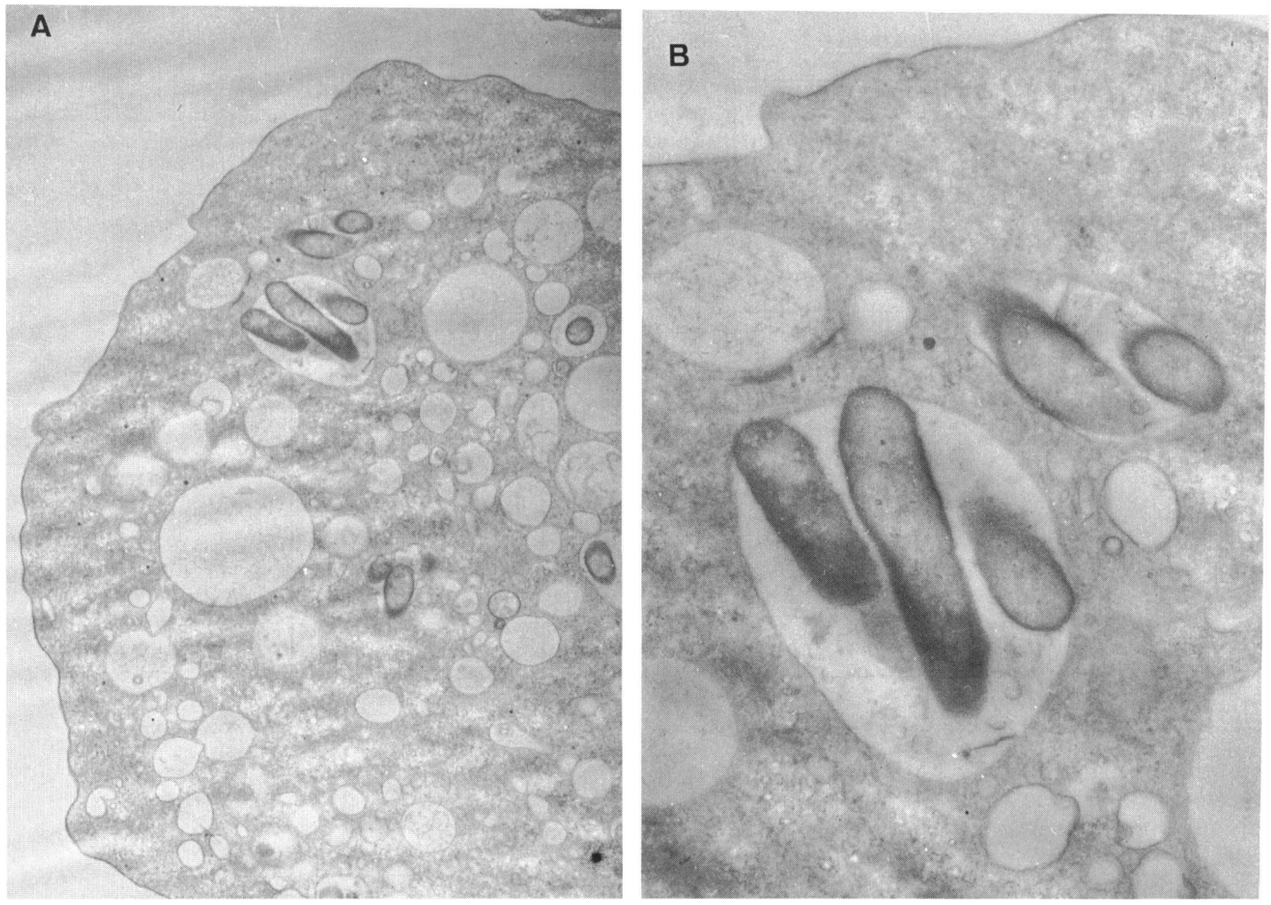

FIG. 2. Electron micrographs of thin sections of amebae that interacted for 30 min at 37°C with (A) *Escherichia coli* 7343. (B) Vacuole magnification. Thin sections were prepared after Epon embedding as described in the text. Bacteria were almost exclusively found inside vacuoles. Reprinted from *Infection and Immunity* (12).

factors found in the liver. That growth of the ameba was supported by viable bacteria (or gamma-irradiated bacteria which have intact proteins), trypanosomes, or vertebrate cells which are phagocytized by the trophozoites suggested that these cells provide essential enzymatic systems which contributed to the metabolic activity of the parasitic cells.

Axenic cultivation or the multiplication of a single microbial species in the absence of other metabolizing cells permits investigations of an order not possible with xenic or even monoxenic (26, 29) cultures because the roles of the associates are difficult to assess. In the case of *E. histolytica*, the development of a culture medium for axenic cultivation turned out to be an ordeal that spanned four decades from 1925, the first time Boeck and Drbohlav (11) announced that they had cultivated *E. histolytica*, until 1961 when Diamond (25) demonstrated unequivocally that *E. histolytica* could be grown in the absence of all other living cells. This breakthrough was achieved after exhaustive search for suitable additives such as vitamins, liver extracts, serum, reducing agents, and other nutrients which enabled the gradual replacement and abolishment of trypanosomes or bacteria from monoxenic cultures (26, 29). However, in spite of this achievement, not all isolates of *E. histolytica* could be axenized, and many strains, especially those isolated from asymptomatic carriers, still require the presence of viable bacteria for in vitro growth (27, 28). Morphological differences exist between xenic and axenically grown trophozoites, and, as discussed in the following sections, other differences may also exist between them.

The development of axenic cultures in which large numbers of trophozoites of various strains could be obtained in a pure form with relative ease has made a dramatic impact on research on this parasite and provided, among other benefits, the basis by which studies of various aspects of the ameba-bacterium-host interrelation, especially with respect to pathogenicity and virulence, could be undertaken.

ATTACHMENT AND INGESTION OF BACTERIA BY TROPHOZOITES OF *E. HISTOLYTICA*

Trophozoites of *E. histolytica* grown in association with bacteria are active feeders that phagocytize numerous bacteria (12, 13, 97). Ultrastructural studies have shown many vacuoles containing bacteria in various stages of digestion. Amebae from axenic cultures that were reassociated with bacteria for short periods also showed ingested bacteria (Fig. 2), and in this respect these trophozoites did not significantly differ from those from monoxenic or xenic cultures.

Studies carried out in our laboratory in recent years have demonstrated that trophozoites of *E. histolytica* are quite selective with respect to their interactions with different bacterial species and only those bacteria which have the appropriate recognition mechanisms will become attached and ingested by the ameba (12, 13). This may be one of the

reasons why certain bacteria were unable to support growth of the ameba in cultures (7, 71). A convenient method for studying the interaction between various types of bacteria and trophozoites based on the differential sedimentation of cells in Percoll density gradients was developed (12). In this system, which is based on the different densities of eucaryotic and procaryotic cells, the rapid separation of amebae from nonassociated bacteria was achieved. Bacteria that adhered to and were ingested by the amebae sedimented together with the trophozoites at a density of 1.06 mosmol/kg, whereas the nonattached bacteria banded at a density of 1.24 mosmol/kg.

The mechanisms by which bacteria adhere to eucaryotic cells (mainly mammalian epithelial cells) have been extensively investigated in recent years (64). In many bacteria, special surface organelles (pili, fimbriae, or flagella) possessing sugar-binding activities (lectins) have been discovered, and these mediate the binding of the microbial cell to carbohydrate-containing receptors present on the epithelial cell (76). Two types of lectin activities have been detected in trophozoites of *E. histolytica*. One of them is a chitotriose-sensitive lectin (45, 63), whereas the other is a galactose/*N*-acetylgalactosamine (Gal/GalNAc)-sensitive one (88, 90). Depending on the conditions used, both lectins seem to be involved in the recognition and mechanism of attachment of trophozoites to other cells (46, 63, 89). The molecular mechanism of bacterial attachment to trophozoites of *E. histolytica* was shown to be mediated by both bacterial (64, 76) and amebic Gal/GalNAc (12, 13, 65) lectins.

Interaction with Mannose-Binding Bacteria

One of the most commonly found bacterial lectins is the mannose-binding substance of numerous gram-negative strains (58, 64, 76, 109). Since trophozoites of *E. histolytica* had been shown to contain receptors for concanavalin A (111), it was logical to assume that adherence of such bacteria to amebae would be mediated by the bacterial mannose-binding lectin which was previously also observed to facilitate bacterial adherence to macrophages (9, 10). The attachment of metabolically radiolabeled *Escherichia coli*, O115 cells, possessing mannose-binding properties, to trophozoites was not significantly affected by temperature but could be inhibited by the presence of α-methyl mannoside (αMM) (1%) in the incubating mixture. At 37°C bacteria attached very rapidly and became mostly irreversibly bound to the amebae, whereas when incubations were done at 5°C or with glutaraldehyde-fixed amebae the bacteria were more easily removed by αMM. Precoating of the mannose receptors on the amebae by concanavalin A also markedly blocked the attachment of the *Escherichia coli* cells. Glutaraldehyde-fixed *Escherichia coli* cells retained their mannose-binding activity, and they readily attached to glutaraldehyde-fixed amebae; this attachment also was efficiently blocked by αMM, and most bacteria which had attached (>90%) could be removed from the amebae by the inhibiting sugar (13) (Fig. 3).

The binding of mannose-binding *Escherichia coli* strains to *E. histolytica* trophozoites was found to be time and concentration dependent. The average number of bacteria that attached per trophozoite during 30 min at a ratio of 1,000 bacteria per ameba was between 40 and 100 bacteria. The attachment did not appear to be homogeneous. Some trophozoites had large numbers of bacteria attached to them, whereas others had only a few. Most ameba had attached bacteria when observed by scanning electron microscopy or

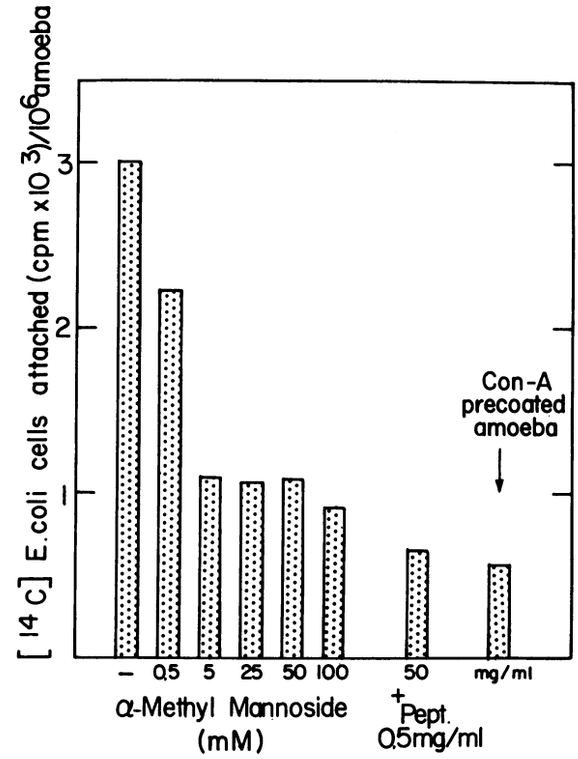

FIG. 3. Effect of increasing concentrations of αMM and precoating of trophozoites with concanavalin A (Con A) on adherence of radioactively labeled *Escherichia coli* to *E. histolytica* trophozoites. The reaction was carried out for 15 min at 37°C. Reprinted from *Infection and Immunity* (12).

by fluorescent microscopy with fluorescamine-labeled bacteria. Thin sections of trophozoites revealed that most also contained ingested bacteria (Fig. 2).

Whether the mannose-binding mechanism described here is functional also in the intestine is not yet clear. Furthermore, the question of appearance and disappearance of the mannose-specific pili (or fimbriae) on the surfaces of bacteria and their role in adherence of the organism to host cells in vivo has not yet been resolved; this is actively being investigated in a number of laboratories (75, 109).

Interaction with Other Bacterial Species

Trophozoites of *E. histolytica* were found to ignore bacteria that did not have a mannose-binding capacity. Thus, various strains of *Shigella* (*Shigella sonnei*, *S. flexneri*, and *S. dysenteriae*) as well as *Bacteroides fragilis*, *Staphylococcus aureus*, and *Micrococcus luteus*, which are known to lack mannose-binding lectins, did not attach at all to the trophozoites. Since *S. flexneri* cells were known to agglutinate with the multivalent concanavalin A lectin, precoating of these bacteria with small concentrations of the multivalent concanavalin A converted them into mannose-binding bacteria that readily attached to the amebae, and this adherence was very sensitive to αMM. Examination of thin sections of amebae that interacted with concanavalin A-coated *Shigella* cells revealed, however, that practically no bacteria were internalized, probably due to cross-linking of surface receptors by the concanavalin A (12).

Phagocytic cells, such as macrophages, are known to attach and ingest opsonized bacteria by virtue of recognizing

the Fc region of the immunoglobulin molecule (47). Coating of the *Shigella* or *Staphylococcus* cells with their specific antisera made possible the attachment and phagocytosis of such bacteria by the amebae and opsonized shigellae were ingested into the ameba vacuoles. This process was dependent on the concentration of the immune serum used and did not occur at low temperature. The mechanism of attachment of opsonized bacteria to the amebae differed, however, from that of macrophages by the fact that Fab' dimers, obtained after pepsin digestion of the immunoglobulin and removal of the Fc-containing fragments by staphylococcal protein A precipitation, were still capable of mediating the adherence of *Shigella* cells to the parasite but not to macrophages (12). Furthermore, a preparation of aggregated human immunoglobulin G, the known Fc inhibitor, which was very efficient in blocking the adherence of opsonized bacteria to macrophages, had very little effect on the binding of opsonized bacteria to the ameba.

Binding of opsonized bacteria to the ameba was found to be mediated by the ameba lectin which recognized galactose or *N*-acetylgalactosamine and galactose residues (13, 90). Both of these sugars are known to be present in the Fc and Fab regions (light chains) of the immunoglobulin molecule (74). Additions of galactose or *N*-acetylgalactosamine markedly inhibited the attachment of opsonized bacteria to the ameba (13).

Further confirmation that the ameba GalNAc-sensitive lectin (88, 89) can be directly involved in attachment of bacteria to the trophozoite was obtained from studies on the attachment of certain bacteria such as *Escherichia coli* serotype O55 or *Salmonella greenside* serotype O50, which lack mannose-binding lectins but possess *N*-acetylgalactosamine as a component of surface antigens (79, 118) (Fig. 4). The attachment of *Escherichia coli* O55 to the trophozoite was inhibited by GalNAc (2 mg/ml) and was independent of the attachment of mannose-binding bacteria so that both strains could be ingested simultaneously. This attachment was, however, affected by the presence of opsonized bacteria such as *Shigella* species, which most likely compete for the same carbohydrate-binding component on the surface of the ameba (13).

As mentioned above, most of the bacteria in the intestinal tract are mixtures of anaerobes and microaerophilic organisms which are difficult to cultivate and identify. Considerable variations exist among different individuals in the composition of their flora at any given time. The bacteria which normally reside in the colon appear to be mostly associated with the intestinal mucus (22, 23, 40, 105). This complex mixture of high-molecular-weight glycoconjugates contains numerous carbohydrate moieties including mannose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine (1, 87). The potential for competitive recognition that the mucus sugar residues may have and their effect on the mechanism of adherence of bacteria to the trophozoite have yet to be determined.

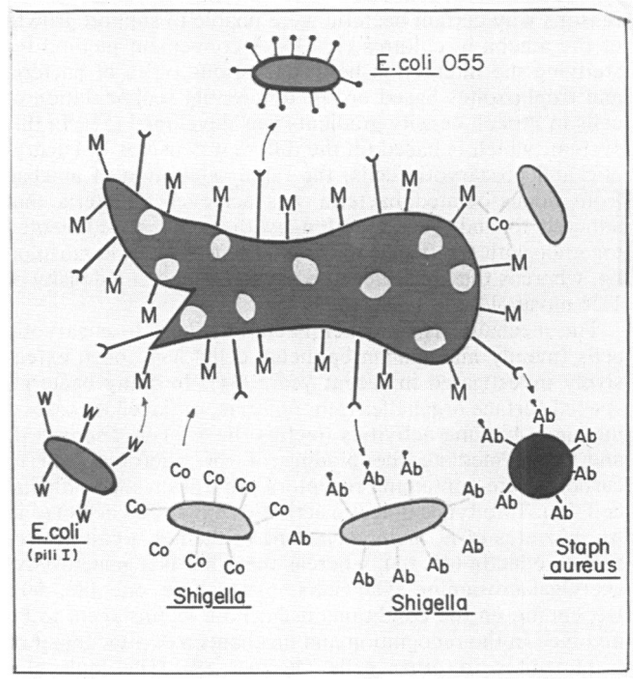

FIG. 4. Summary of various possibilities of attachment of different bacteria to amebic trophozoites. M, Mannose-containing receptors on the trophozoite surface; Y, carbohydrate-binding (lectin) activity; W, *Escherichia coli* surface pili which have mannose-binding properties; Co, concanavalin A bound to receptors on the bacteria; Ab, antibodies; ¶, *N*-acetylgalactosamine or galactose on bacterial surfaces or on opsonins. Reprinted from *Infection and Immunity* (13).

Ingestion of Bacteria

The role of ingestion of bacteria by trophozoites depends on the concentration and ratio of bacteria and amebae. At high multiplicity (1,000:1) the ingestion is very rapid and can reach 100 bacteria per h (12). Ultrastructural studies of thin sections of trophozoites have shown that, as early as 10 min after the association of axenically grown trophozoites with suitable bacteria, many vacuoles contain one or more bacteria. Another indication that the ameba internalized and ingested the bacteria was obtained by subjecting trophozoites that had interacted with metabolically radiolabeled bacteria to the action of mild detergents. Similar to what was previously found with the *Dictyostelium* amebae (112), Triton X-100 (0.2%) rapidly disrupted the *E. histolytica* trophozoites together with the phagocytized bacterial cells (12). Unattached bacteria or those that remained attached to the outer surface of the trophozoite, as shown by experiments done at 5°C (when there is no phagocytosis), were not dissolved by the detergent, and over 95% of their radioactive content was recovered in the intact bacteria. Once a bacterium is inside the ameba, its cell wall very rapidly loses its property as a permeability barrier and the bacteria lyse. The sensitivity to detergents could be detected as early as 15 min after the association of bacteria and amebae (12). No degradation of bacteria was observed, however, in the case of concanavalin A-coated *Shigella* cells, which remained attached only to the surface of the trophozoites. The mechanism by which the degradation of the bacterial cell wall occurs in the ameba vacuoles is not yet known. It could involve acidic and chelating molecules, as well as lytic enzymes, which most likely cause the collapse and disintegration of the structural arrangement of the membrane layers in the bacterial cell wall envelope.

In summary, our studies indicate that *E. histolytica* is quite selective with regard to its interactions with bacteria, and it has a variety of options by which to bind and ingest them (Fig. 4). The ability of the trophozoite to interact and digest bacteria is apparently important for the growth of the parasite and its pathogenicity (see below).

EFFECTS OF BACTERIA ON AMEBIC VIRULENCE

The study of experimental amebiasis suffers from the unavailability of a suitable animal model. Humans, and perhaps some primates, are the only natural host in which ameba cysts are formed and the three states of amebiasis (commensalism, intestinal tissue invasion, and extraintestinal lesions) occur (16). Partial animal models exist. Some kinds of New World monkeys have been reported to harbor the parasite, and some amebic infections can be induced in various animal models such as hamster liver abscesses (30, 71, 95), kitten diarrhea (19), and rabbit or guinea pig cecal ulcerations (72, 84, 86, 110). Unfortunately, none of these systems is very reproducible or quantitative.

An isolate of *E. histolytica* derived from a symptomatic case of human amebiasis may be regarded as potentially pathogenic to a suitable host when introduced orally in cyst form or intraintestinally as trophozoites (33). Virulence, on the other hand, is the measure of the degree of pathogenicity so that, given different conditions, the same strain of *E. histolytica* may sometimes vary widely in its virulence. The pathogenic activity of amebae seems to depend on an inherent potential within the parasite and the provision of a suitable host. Such inherent potential appears to be hereditary in amebae. What needs to be determined is the mechanism by which virulence can be enhanced or suppressed.

The definitive influence exerted by bacteria in the production of amebic dysentery has been observed repeatedly (14, 24, 83–85, 117, 119). Luttermoser and Phillips (52) found that the infectivity and pathogenicity of cultures of *E. histolytica* strain 200 varied depending on the culture microbial associates. When cultivated with rabbit intestinal flora, trophozoites produced acute disease in most of the infected animals. However, much less virulence was found if the ameba were cultured with organism t, an undefined anaerobic bacillus, or with trypanosomes (82, 85). Reassociation with rabbit intestinal flora returned their infectivity.

Other investigators have shown that adding pathogenic streptococci to the inocula of amebae decreased the incubation period of the experimental disease in kittens and mortality increased (85, 113). In vitro grown amebae that had lost their virulence and were incapabe of producing experimental disease recovered their virulence upon addition of pathogenic bacteria, the most effective bacteria being *Escherichia coli*, *Salmonella typhosa*, and *Salmonella paratyphi* (24, 83). These results have led to the generally accepted conclusion that the type of bacterial associates with which the amebae are grown in vitro probably constitutes a major factor that should be considered when evaluating the infectivity and pathogenicity of strains of *E. histolytica*.

An interesting, although somewhat controversial, observation was made by Westphal (117) in 1937: he ingested a pure culture of amebae and was able to demonstrate the presence of the parasite in his own feces 2 days later. He remained, however, asymptomatic for a long period, after which he and an equally brave colleague ingested bacteria from a patient with amebic dysentery. After a few days Westphal suffered an episode of amebic dysentery. It is not certain what this heroic experiment proved, but it is in accordance with the above-mentioned and better-controlled observations that attest to the important role of bacteria in determining the pathogenicity of the parasite.

Enhancement of Virulence in Axenically Grown Amebae

Analysis of enhancement of virulence in axenically grown amebae was hindered for many years due to inability to grow amebae without bacteria in vitro. But since this was accomplished by Diamond (25, 26), a number of investigators have shown that axenically grown trophozoites of *E. histolytica* gradually lose their virulence upon prolonged axenic cultivation (56, 81) and regain it when trophozoites are passaged through the hamster liver (51, 73). The first studies which confirmed that bacteria could enhance virulence of axenically grown trophozoites of *E. histolytica* were done by Wittner and Rosenbaum (119). These investigators found that reassociation of trophozoites with *Escherichia coli* for at least 6 h before inoculation into the hamster liver dramatically increased the percentage of animals with hepatic abscesses. Heat-killed bacteria, crude bacterial homogenates, or media in which bacteria had been grown proved ineffective in causing increased virulence of axenically grown *E. histolytica* trophozoites. Although these results suggested that a factor provided by living bacteria may be required to sustain the virulence of the amebae, no further studies were made at that time to elucidate the biochemical mechanism by which the ameba-bacterium interaction and the ensuing stimulation of virulence occurs.

In recent years considerable progress has been achieved in the development of in vitro experimental assays for determining amebic virulence. Good correlations have been obtained between the various in vivo and in vitro assays, and comparisons between several axenically cultivated strains have shown that pathogenic strains such as HM-1:IMSS very readily (i) produce hepatic lesions in hamsters (30, 36), (ii) lyse monolayers of tissue-cultured mammalian cells (14, 55, 78), (iii) phagocytize erythrocytes (77), and (iv) kill human macrophages and polymorphonuclear neutrophils (39, 88, 98). Using the destruction of tissue culture monolayers as a semiquantitative in vitro assay for determining the virulence of amebae, we recently found that short-term association of axenically grown *E. histolytica* trophozoites with bacteria significantly stimulated their activity (14). Preincubation of ameba trophozoites of strain HK-9 for short periods (up to 1 h) with increasing amounts of an *Escherichia coli* strain that had a surface lectin specific for mannose residues (76) gradually increased the rate of destruction of monolayers of tissue-cultured mammalian cells. Maximal destruction rates were observed when the ratio of trophozoites/bacteria was 1:1,000, although at a ratio of 1:10 a small increase in the rate of destruction above that of trophozoites without bacteria could be seen. Bacteria alone had no effect on the monolayers of tissue-cultured cells (Fig. 5). The addition of αMM (10 mg/ml) to the bacterium-ameba mixture inhibited the bacterial lectin and prevented the attachment of the bacteria, and consequently also the increase in amebic virulence.

Increased amebic virulence was observed after reassociation not only with bacterial strains that possess mannose-binding lectins (58), but also with other types of bacteria such as *Escherichia coli* serotype O55 (13) that were found to attach to the amebae by virtue of having cell surface carbohydrates that serve as binding sites for the ameba lectin (see above). In these cases, galactose or lactose (5 mg/ml) inhibited both the interaction and damage of the trophozoite to the mammalian cell (88, 90) and its ability to attach and ingest the bacterial cells (13). To be an effective inhibitor, galactose (or GalNAc) had to be present before the addition of the bacterial cells.

The possibility that the bacteria increased amebic virulence because they contained plasmids or virulence factors was ruled out by the use of *Escherichia coli* cells which were chemically cured or devoid of plasmids (14). As long as the

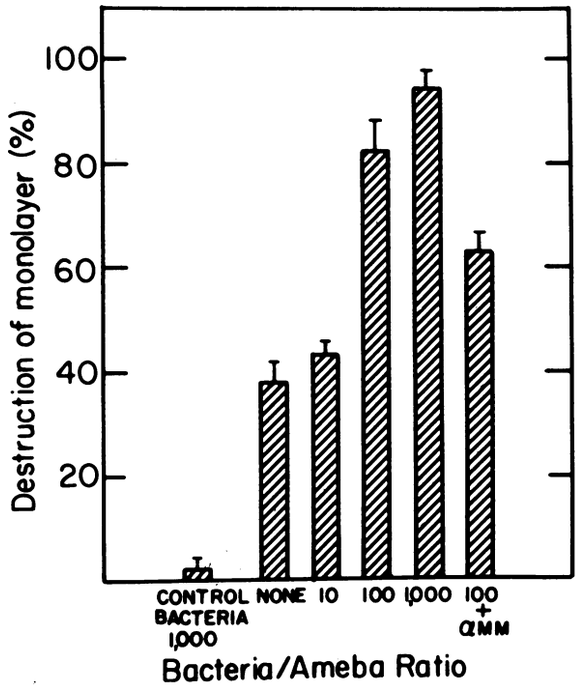

FIG. 5. Effect of associated bacteria on rate of destruction of tissue culture monolayers by trophozoites of *E. histolytica*. Various amounts of *Escherichia coli* O115 cells were preincubated with *E. histolytica* strain HM-1:IMSS (2×10^5 cells per ml) for 15 min, after which the incubation mixture was added to monolayers of BHK cells at a trophozoites/BHK cell ratio of 1:1 and further incubated for 60 min. Determination of mammalian cells remaining in the wells after the incubation was by staining with methylene blue and acid extraction of the dye. Controls containing only bacteria (2×10^8) were also incubated. To one of the experiments (at a ratio of 100 bacteria per ameba) αMM (0.5% final concentration) was added at the beginning of the incubation to inhibit the attachment of the *Escherichia coli* cells to the trophozoites (12). Reprinted with permission from the *Journal of Experimental Medicine* (14).

bacteria possessed the necessary recognition mechanisms (lectins or receptor for amebic lectin) and attached to the amebae, they stimulated amebic virulence.

Association of amebae with bacterial strains which are incapable of attachment or ingestion by the trophozoites (12), such as the anaerobic bacteria *B. fragilis* and the gram-positive bacteria *Staphylococcus aureus* and *M. luteus* did not stimulate their virulence. A slight augmentation of virulence was observed upon association of amebae with opsonin-coated *Shigella* spp., conditions that enabled attachment and ingestion of the bacteria by the trophozoites (14). Enhancement of amebic virulence after association with bacteria was detected with several axenically cultured *E. histolytica* strains (HK-9, HM-1:IMSS, and 200:NIH).

Association of axenically grown trophozoites of *E. histolytica* with bacteria not only stimulated the destruction of a variety of tissue-cultured cell lines, but as previously reported, also facilitated the formation of hepatic abscesses in hamsters (119) and enabled the penetration of the guinea pig colonic mucosal layers (62). Furthermore, bacteria-associated trophozoites displayed higher rates of erythrophagocytosis (Garcia Rivera et al., *in* Xth Seminar on Amebiasis IMSS, Mexico City, p. 24, 1986) and more efficiently killed human polymorphonuclear neutrophils and macrophages (Salata et al., unpublished data).

Virulent strains of *E. histolytica* have been shown to contain a variety of soluble toxic substances such as an enterotoxin (34, 50), a variety of collagenase and proteolytic enzymes (35, 38, 57, 69), and a protein with ionophore-forming capabilities (amebapore) (53, 120). Axenically grown trophozoites which were associated with bacteria for periods of 1 to 2 h had higher levels of toxic substances (34). This was particularly evident in strains with attenuated virulence such as HK-9, in which after association with *Escherichia coli* cells the levels of the enterotoxin became similar to those present in more virulent strains such as HM-1:IMSS. Interestingly, the level of the activity of the amebapore did not increase following interaction with the bacteria (Rosenberg et al., unpublished data).

Requirements for Virulence Stimulation

For amebic virulence to be stimulated by bacteria, certain conditions were required. Maximal augmentation of virulence was observed when preincubations between trophozoites and bacteria were between 15 min and 1 h. No difference was found when using log- or stationary-growth-phase bacteria or with bacteria grown in rich or minimal media. On the other hand, heat-inactivated, glutaraldehyde-fixed, or sonicated bacteria did not stimulate virulence, nor did the bacterial cell envelope or cytosol fractions obtained after mechanical disruption of bacteria. Stimulation was observed, however, with bacteria that had been exposed to a lethal dose of gamma radiation from a cobalt source (500 krad) (14). This is in contrast to the observations of Wittner and Rosenbaum (119), who found that irradiated bacteria were incapable of stimulating hepatic abscess formation. This discrepancy could perhaps be attributed to the difference in the strains of bacteria and amebae used and, in our case, to the high ratio of bacteria/amebae and the use of the bacterial cells immediately after their lethal irradiation. Another difference was in the assays used to determine virulence. Liver abscess formation was determined after 14 days and involves continuous interaction with host defenses, whereas the in vitro assay uses monolayers of tissue-cultured cells in which the interaction is for only 1 to 2 h.

Bacteria grown for three generations prior to harvest with cell wall synthesis inhibitors such as cephalexin or bicyclomycin, which induced morphological alterations (filamentous form), were capable of adhering to the trophozoites and stimulating their virulence. On the other hand, pretreatments of bacteria with protein synthesis inhibitors such as amikacin or nalidixic acid, which markedly affected bacterial growth but did not cause lysis, decreased their ability to stimulate virulence. None of these antibiotics, when added to axenically grown cultures of *E. histolytica*, had any effect on amebic virulence or growth (14).

BACTERIAL CONTRIBUTION TO AMEBIC ACTIVITIES

Trophozoites dwell in the colon in the presence of a variety of bacterial species, and many of these were found to exert a strong chemoattractant effect on the amebae (6). For many years it was believed that bacteria served as a nutrient source for the ameba and provided a suitable gas mixture composition for growth. Results obtained in recent preliminary investigations confirm earlier observations (81, 82) that the associated bacterial flora may also be essential for the encystation and excystation of *E. histolytica* cysts (D. Mirelman, unpublished results). That in vitro ingestion of certain types of bacteria by trophozoites also rapidly stimulated a

virulent response in the parasite suggested that the bacterial contribution may be related more to the anaerobic respiration system of the amebae than to a direct transfer of bacterial genetic information. Although *E. histolytica* trophozoites slowly die in the presence of atmospheric conditions, they are considered microaerophilic organisms and are known to have an affinity for oxygen, some of which they consume and metabolize into water (8, 37, 49, 115). Substrate oxidation and electron transfer pathways of *E. histolytica* have been investigated to some extent and found to include a ferredoxin molecule and probably another iron-sulfur protein (116). Trophozoites, however, have been shown to contain virtually no catalase, have low levels of superoxide dismutase (70), and require low redox potentials for growth (26, 28, 37).

Effects of Microaerophilic Conditions and Oxidized Molecules

Successful anaerobic cells must protect themselves from a number of highly reactive species of molecules and radicals generated directly or indirectly during the reduction of oxygen. Products toxic to anaerobic organisms are known to build up rapidly in culture media exposed to air and may actually arise from agents added to the medium to lower the oxidation-reduction potential (18, 67). These reactive products may slowly damage cell components such as sulfhydryl and metalloprotein groups, cause a rise in the oxidation-reduction potential of the cell, and inhibit the electron transport, thus consuming the reducing power of the cell.

To investigate the possible role that the above-mentioned toxic by-products may have on amebic virulence, experiments were carried out also under microaerophilic conditions in jars containing gas-generating kits designed for campylobacters, which are devoid of toxic hydrogen gas (8). The results show that the virulence of trophozoites increased considerably under such microaerophilic conditions and the rates for mammalian cell destruction approached those observed with associated bacteria in normal ambient (14).

Hydrogen peroxide has been shown to be very toxic to amebae (70). In the presence of hydrogen peroxide (>100 μM), trophozoites lost their ability to damage tissue-cultured cells (14). Trophozoites that had ingested bacteria seemed to be protected from these effects and showed an augmented virulence which was not inhibited even in the presence of relatively high concentrations of H_2O_2 (1 mM). Association of trophozoites with bacterial strains deficient in catalase such as *S. dysenteriae* type I or a mutant such as *Escherichia coli* CAT 4 (14), which contains <10% of the catalase activity present in the wild-type *Escherichia coli* K-12, stimulated amebic virulence, even at low bacteria/trophozoites ratios. These bacteria, however, afforded less protection to the ameba and virulence was already impaired at 200 μM H_2O_2. Although these bacteria were devoid of catalase, they still were capable of destroying hydrogen peroxide at slower rates, probably by virtue of other peroxidases or metal-containing components which enable nonenzymatic reactions. Direct incubation of the ameba with bovine catalase did not cause the uptake of the enzyme and the cells were not protected from H_2O_2 (14).

Associated bacteria are apparently also useful in destroying other oxidized molecules such as superoxide radicals which are toxic to the ameba (70). Ingested bacteria abolished the inhibitory effects of methyl viologen (Paraquat) (14), a compound known to stimulate the production of toxic superoxide ions (41). Evidence for an increase in the reducing power of the trophozoites that had ingested bacteria was obtained also from the enhanced capacity to reduce exogenously added cytochrome *c* (R. Bracha and D. Mirelman, unpublished results) and nitrotetrazolium blue (5).

Effects of Metronidazole

One of the classes of drugs that have a marked specific toxic effect on amebic trophozoites (and other anaerobic microorganisms) are the 5-nitroimidazoles, and one of the most commonly used in clinical situations is metronidazole (Flagyl). This class of compounds is apparently reduced to a free radical species by the amebic ferredoxin molecules, and the toxic radicals produced subsequently inhibit DNA and protein synthesis (68). In vitro exposure of trophozoites to metronidazole at a concentration of 50 μg/ml for short periods (up to 1 h) had no significant effect on the basal level of virulence of amebae. The presence of metronidazole, however, markedly inhibited the stimulation of virulence that occurs after association with bacteria or anaerobic conditions (14). Moreover, the uptake of ^{14}C-labeled metronidazole by trophozoites of *E. histolytica* was markedly enhanced in the presence of bacteria. Only live or gamma-irradiated bacteria, which are known to interact and stimulate amebic virulence, increased the uptake of metronidazole. Since the uptake of metronidazole molecules by trophozoites is known to be a function of their rate of reduction by the ferredoxin (68), the increased in vitro uptake, in the presence of bacteria, was a clear indication of an acceleration in the cell's electron transport system or an increase in the reducing power of the cell.

At first glance, these results are intriguing and difficult to interpret. Metronidazole is known to be clinically more effective against amebae in tissue (where bacteria are not present) than luminal amebae, despite high levels of drug in stools. Furthermore, xenic cultures of *E. histolytica* grown together with their accompanying bacterial flora appear to be slightly less sensitive to metronidazole than axenic cultures (Mirelman, unpublished observations). This apparent inconsistency with our in vitro results may be due to the nature of the accompanying intestinal bacterial flora, which is mostly anaerobic and devoid of detoxifying enzymes and thus cannot stimulate amebic respiration. Moreover, in contrast to the aerobic organisms used in our in vitro study, the anaerobic bacteria which cohabit with the amebae in in vivo conditions contribute, with their own systems, to the reduction and elimination of metronidazole molecules needed for the killing of amebic trophozoites.

In conclusion, our recent results lend further support to the hypothesis that virulence of a given pathogenic *E. histolytica* strain may depend to a considerable extent on the activity of its electron transport system or the cell's reducing power. Both anaerobic conditions and the ingested baceria (especially aerobic ones) apparently favor the lowering of the redox potential in the ameba cell. Both appear to facilitate the electron transort system of the ameba: the first by virtue of oxygen deprival (66), and the latter apparently serving as broad-range scavengers for toxic oxidized molecules which inhibit the anaerobic respiration. Which of the various bacterial enzymatic systems, components, or products is responsible for this effect is not yet completely clear. Iron-binding proteins of the bacteria such as enterochelins (48) and cytochromes, as well as proteins rich in sulfhydryl groups, may serve as electron donors and contribute by maintaining the reduced state of the trophozoite. Furthermore, although no conclusive evidence has been obtained as

yet in our studies on the direct contribution of bacterial catalase and superoxide dismutase to amebic virulence, it would be premature to discount their possible participation in protecting the trophozoites from highly toxic oxidized metabolites.

EFFECT OF BACTERIAL ASSOCIATES AND CULTURE CONDITIONS ON ISOENZYME PATTERNS (ZYMODEME)

Another influence that the bacterial associates appear to have in *E. histolytica* is with respect to the electromobility properties of certain amebic isoenzymes. Analysis of the electrophoretic mobility of various isoenzymes has been gaining popularity as a means for distinguishing between different strains and species of *Entamebadidae* (93, 100, 101, 104). Studies on isoenzyme patterns (zymodemes) of *E. histolytica* isolated from thousands of asymptomatic and symptomatic human cases have resulted in the characterization of at least 20 distinct zymodemes (99–101). Based on the clinical picture of the source of the isolate, these zymodemes are placed in one of two categories: pathogenic or nonpathogenic. Two isoenzyme systems, the hexokinase and phosphoglucomutase, have been found to differ in their electrophoretic migration in pathogenic and nonpathogenic *E. histolytica* (Fig. 6). The nonpathogenic isoenzyme patterns, with rare exception, are obtained in isolates from asymptomatic carriers with negative serology, while the pathogenic zymodemes, which are the minority of cases, are usually derived from individuals with positive serology and presence or history of some clinical symptoms. These findings have focused renewed attention to the old observations of Brumpt (17), who postulated two species of *E. histolytica* that are morphologically identical. One of them, labeled *E. dispar*, is nonpathogenic and incapable of causing disease, while the second, *E. dysenteriae* is pathogenic and capable of causing disease (invasion) in humans. The finding of a nonpathogenic zymodeme in conjunction with a pathogenic one in any single host has never been reported (99, 103). Furthermore, no evidence of alteration of isoenzyme patterns, i.e., shifts from nonpathogenic to pathogenic or vice versa, was ever demonstrated in longitudinal culture studies (99, 103) which were conducted in the presence of viable bacteria in Robinson's medium (96). Moreover, short-term interaction (for 1 h) of axenically grown ameba with bacteria, which markedly enhanced their virulence (see above) (14), did not cause any changes in their pathogenic zymodeme (60). The electrophoretic bands belonging to the bacterial isoenzymes did not interfere or affect in any case the readings of the patterns given by the amebae (60, 103). Based on all of these studies, it has been suggested that zymodemes can be used as biochemical markers to distinguish between the two different *E. histolytica* species and that persons harboring amebae with nonpathogenic zymodemes may not require treatment (31, 102).

Little progress has been made towards our understanding of the differences at the molecular level between *E. histolytica* isolates that possess pathogenic zymodemes and those which have nonpathogenic ones. This has been mainly due to the unavailability of any axenic strains of the latter, as in contrast to pathogenic isolates, all attempts to cultivate nonpathogenic amebae under axenic conditions failed. This feat has recently been accomplished (59); propagation of a nonpathogenic ameba, *E. histolytica* CDC strain O784:4, could be done in a medium for axenic cultivation (29)

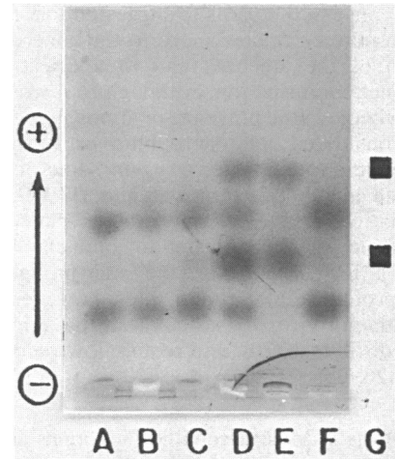

FIG. 6. Electrophoretic pattern of hexokinase isoenzymes from *E. histolytica* SAW 1734R clAR. Trophozoite samples were harvested from each growth condition, and lysates were prepared and applied to agarose minigels (59). A, Nonpathogenic migration pattern of *E. histolytica* SAW 1734R clAR cultured together with their accompanying bacterial flora; B, nonpathogenic pattern of SAW 1734R clAR 17 days after transfer to a medium for axenic growth (TYI–S-33) (29) supplemented with gamma-irradiated bacteria (59); C, nonpathogenic pattern of cultures 18 days after transfer to TYI–S-33 medium (notice the beginning of a pathogenic pattern too); D, trophozoite sample taken 20 days after transfer to TYI–S-33 medium (notice that this culture already contains a mixture of nonpathogenic and pathogenic pattern of isoenzyme); E, pathogenic pattern of trophozoite sample of *E. histolytica* SAW 1734R clAR 21 days after transfer to TYI–S-33 medium; F, nonpathogenic pattern of hexokinase from *E. histolytica* CDC:0784:4 (group I) used as nonpathogenic marker; G, pathogenic pattern from *E. histolytica* HM-1:IMSS used as a pathogenic marker. Samples containing lysates of bacterial cells without ameba did not show any hexokinase bands in this region.

provided that it contained a mixture of antibiotics to suppress the growth of the associated bacterial flora and a nutritional supplement consisting of lethally irradiated bacterial cells. Under these new culture conditions the trophozoites gradually increased their growth rate, and 30 days later the supplement of irradiated bacteria was no longer required. Although at this stage the cultures were not yet completely axenic and the presence of a small number of bacteria ($<10^5$/ml) could still be detected in sterility tests, a change was observed in the morphology of the trophozoites from a relatively smooth endoplasm with few vacuoles to one which possessed many small vesicles, as is usually seen in axenic cultures. At this same time a change was noted in the virulent properties of the trophozoites, as they became capable of inducing hepatic abscesses in hamsters and of destroying monolayers of tissue-cultured cells (14). The most unexpected alteration, however, was in the isoenzyme electrophoretic pattern of the amebae, which changed from nonpathogenic to pathogenic (59). Continuous treatment of the cultures with a variety of antibiotics finally yielded axenic amebae (L. S. Diamond, personal communication), and these retained the newly acquired pathogenic zymodeme.

Zymodeme Conversions in Cloned Cultures of Amebae

Two possible explanations were offered for the observed shifts to pathogenic zymodeme obtained with *E. histolytica*

CDC O784:4 when grown in a medium for axenic cultivation (59). The changes could be ascribed to a previously unknown alteration within the amebae. Alternatively, if the original amebic isolate consisted of a mixed population of zymodemes, the conditions of growth could select for one or the other of the population. To exclude the latter possibility, the experiment was repeated with a cloned culture of a nonpathogenic strain (SAW 1734R clAR, zymodeme group III) isolated by and obtained from P. G. Sargeaunt. The results obtained with the cloned culture were similar to those obtained in the previous experiment and confirmed that an ameba possessing a nonpathogenic zymodeme and nonvirulent properties can change to pathogenic characteristics upon change in growth conditions (61).

The question that remained open was whether pathogenic zymodemes could convert or revert back to nonpathogenic. This was recently accomplished (D. Mirelman, R. Bracha, A. Wexler, and A. Chayen, *in* Xth Seminar on Amebiasis IMSS, Mexico City, 1986) too by reassociating and subculturing in xenic conditions (27) the axenically grown and newly converted pathogenic amebae with the bacterial flora that accompanied this strain in the original xenic culture. A change to nonpathogenic zymodeme was observed after 7 days. No alteration in the pathogenic zymodeme was obtained, however, when the reassociation of the newly converted pathogenic amebae was done with bacterial flora that accompanied a pathogenic strain of amebae, *E. histolytica* SAW 408, isolated from a symptomatic case (91). In both cases and similarly to what was previously shown (60, 103, 104), the electrophoretic migration of the bands from the bacterial isoenzymes appeared in other regions of the gel and did not interfere or affect the reading of the patterns given by the amebae.

At the moment we do not have a rational explanation for the many types of *E. histolytica* zymodemes that have been characterized to date or for the mechanism of conversion seen in our present experiments. One of the reasons why such a conversion may not have been observed until today in any of the longitudinal studies done by Sargeaunt and colleagues (99, 103) may be due to the way the cultures were grown in Robinson's monoxenic media (96). In this system, stool samples are inoculated together with erythromycin to suppress the growth of the original fecal bacterial flora, while separately grown *Escherichia coli* B cells are added to the cultures. The addition of these particular *Escherichia coli* cells to the growing cultures supports their growth but apparently does not cause any changes in their zymodeme. In our experiments, changes in isoenzyme patterns were obtained when the bacterial flora was almost completely eliminated during the lengthy process of axenization. These conditions were undoubtedly quite stressful for the amebae; as a consequence, changes in metabolic systems may have occurred which could also cause alterations in isoenzyme patterns.

The data obtained until now suggest that the isoenzyme patterns of *E. histolytica* depend on the culture conditions of the trophozoites, especially in the nature of the associated bacterial flora. Some types of bacteria and perhaps also rich axenic media may favor the induction and appearance of "pathogenic" isoenzyme patterns in the ameba, whereas others, such as the flora found in asymptomatic carriers, may cause the development of "nonpathogenic" ones.

E. histolytica is not the first organism in which isoenzyme electrophoretic patterns have been found to change. Somewhat similar alterations have been shown to happen in other protozoa (*Tetrahymena* and *Paramecium* spp.) in which variations in isoenzyme patterns are subject to conditions of growth, media components, and the presence or absence of bacteria in the medium (1–3). In *E. histolytica* the shift to pathogenic zymodeme appears to coincide with the capability of the ameba to become virulent, and this could be of extreme importance for development of symptomatic amebiasis in humans. Moreover, the reversion from pathogenic to nonpathogenic ameba is of great interest as it may help us understand the multitude of asymptomatic cases.

The isoenzymes which serve as indicators of *E. histolytica* pathogenicity (hexokinase and phosphoglucomutase) are enzymes of the glycolytic pathway and do not seem to be virulence factors or have a direct role in the pathogenic mechanisms of the amebae. They appear, however, to serve as useful biochemical markers which enable us to follow the conversion from one state to the other.

Investigations on the structural modifications in the isoenzyme polypeptide chains which cause the differences in their electrophoretic migration are under way. These could help to clarify their importance for strain classification and shed more light on the molecular mechanisms of interconversion. Furthermore, they may indicate whether the isoenzyme shifts are of a phenotypic or a genotypic nature.

CONCLUDING REMARKS

The evidence for the many influences of bacterial flora to the well-being of the ameba is compelling. Perhaps the intriguing and potentially important effect seems to be the ability of certain bacterial species to switch on the pathogenic and virulent potential of the trophozoite, while others have no effect or may even cause them to become avirulent. Our findings support some of the early observations of Hoare (42) and Elsdon-Dew (33), who suggested that instead of two species (*E. dispar* and *E. dysenteriae* [17]) there may actually be two states or phases of the same organism, an active state which manifests aggressive properties and an inactive state, and under certain conditions these states appear to be interconvertible (59, 61).

It might be possible that the bacterial flora present in the intestine of asymptomatic carriers helps maintain the conditions that lead to the development of the nonactive *E. histolytica* state or phase, whereas a change in the bacterial flora caused perhaps by a secondary bacterial (aerobic?) infection or the use of antibiotics may sometimes induce the conversion to the active state which causes the clinical disease. This hypothesis may also explain the observations of Westphal (117), who infected himself with *E. histolytica* but became sick only after also ingesting the bacterial flora of the patient. Considerable variations exist in the nature of the bacterial flora in different individuals and alterations in the human intestinal flora are known to occur, but this has been accepted more by reiteration than by demonstration (105). It would be most interesting to verify whether changes in the microenvironmental conditions of the intestine affect the activities of the amebae.

In principle, we support the notion that amebae in the pathogenic state are capable of causing disease in humans. However, contrary to the established hypothesis and previous beliefs (31, 60), classification of *E. histolytica* isolates as pathogenic or nonpathogenic based on their isoenzyme electrophoretic patterns and the clinical picture of the patient may be of little value. Our recent results (59, 61) clearly demonstrate that isoenzymes of cloned cultures can change from one state to the other. Although no evidence is available that indicates that such changes may also occur some-

times in the microenvironment of the human intestine, one can assume that the potential for this at least exists.

The issues raised by the recent findings deserve further detailed studies, and many questions remain to be answered. One of the pressing and important ones is what should be done in cases where trophozoites with nonpathogenic zymodemes are detected in feces of asymptomatic carriers. Would it be advisable to recommend not to treat asymptomatic carriers harboring trophozoites with nonpathogenic isoenzyme patterns, and inform these patients that the parasite with which they are infected would not cause them disease? At the moment, there is no definite answer and this decision should be left to the discretion of the attending physician.

There is no doubt that further progress in the understanding of the intriguing relationship between bacteria and amebae, as well as on the regulation of conversion from nonpathogenic to pathogenic organisms and vice versa, will probably come from gene cloning and comparisons between the two by using recombinant techniques, as well as from analysis of expression products. It is hoped that results from these studies may allow us in the future to better control the parasite and the widespread disease it causes.

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